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Title: MULTIPLE VIRAL REPLICON CULTURE SYSTEMS

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## MULTIPLE VIRAL REPLICON CULTURE SYSTEMS

### Background

#### (1) Field of the Invention

**[ 0001 ]** The present invention generally relates to methods and compositions for screening for antiviral compounds using cell cultures. More specifically, the invention relates to the use of multiple cell cultures harboring multiple subgenomic viral replication systems to evaluate compounds for antiviral activity.

#### (2) Description of the Related Art

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**[ 0002]** Primary screening programs to discover and identify compounds with antiviral activity can be designed in a variety of ways. All programs, however, fall into one of two general approaches. In the targeted approach, one particular biochemical target is chosen and candidate antiviral compounds are screened for inhibition of that target. The target is often an enzyme or a receptor that is known or thought to be essential to the process of viral replication. The alternative approach is unbiased such that inhibitors of viral replication are sought without *a priori* concern for the target. This unbiased approach generally involves use of cell culture since, as obligate intracellular pathogens, viruses can only replicate within cells. Although cell-based screening has been used successfully throughout the drug-discovery field, it is problematic when screening for antivirals. This is because it requires inoculation of infectious virus onto the cells and the production of additional infectious progeny virus. In particular, handling such infectious material is not easily compatible with the high throughput process of screening large libraries of compounds.

**[ 0003]** Thus, there is a need for improved methods and compositions that are useful for screening and analyzing antiviral compounds. In particular, these methods and compositions should be useful for high-throughput antiviral screening. The invention described herein satisfies that need.

### Summary of the Invention

**[ 0004]** Accordingly, the present invention provides methods and compositions utilizing subgenomic viral replication systems to evaluate potential antiviral compounds. The use of these methods and compositions allows rapid screening of the potential antiviral compounds against multiple viruses simultaneously.

**[ 0005]** In some embodiments, the present invention is directed to methods of screening a candidate antiviral agent for antiviral activity. The methods comprise the use of at least two subgenomic viral replication systems that are genetically distinct from each other. The methods involve preparing a first cell culture comprising cells containing a first subgenomic viral replication system, and a second cell culture comprising cells containing a second subgenomic viral replication system, adding the candidate antiviral agent to each cell culture, incubating the cell cultures under conditions and for a time sufficient to detect an antiviral effect by the candidate antiviral agent on the subgenomic viral replication systems, and determining the effect of the candidate antiviral agent on each viral replication system.

**[ 0006]** Related embodiments of the present invention are directed to methods of screening a candidate antiviral agent for antiviral activity. These methods also comprise the use of at least two subgenomic viral replication systems that are genetically distinct from each other. These methods include combining a first cell culture comprising cells containing a first subgenomic viral replication system and a second cell culture comprising cells containing a second subgenomic viral replication system to make a mixed cell culture, adding the candidate antiviral agent to the mixed cell culture, incubating the mixed cell culture under conditions and for a time sufficient to detect an antiviral effect by the candidate antiviral agent on the subgenomic viral replication systems, and determining the effect of the candidate antiviral agent on each viral replication system.

**[ 0007]** The present invention is also directed to mixed cell cultures that include a first cell culture comprising cells containing a first subgenomic viral replication system and a second cell culture comprising cells containing a second subgenomic viral replication system.

#### Brief Description of the Drawings

**[ 0008]** Figure 1 shows two graphs summarizing results from experiments demonstrating that Sindbis virus replicons cultured separately (top) have similar sensitivity to  $\alpha$ -interferon as Sindbis virus replicons cultured with hepatitis C virus replicons and yellow fever virus replicons (bottom).

**[ 0009]** Figure 2 shows two graphs summarizing results from experiments demonstrating that yellow fever virus replicons cultured separately (top) have similar sensitivity to  $\alpha$ -interferon as yellow fever virus replicons cultured with hepatitis C virus replicons and Sindbis virus replicons (bottom).

**[ 0010]** Figure 3 shows two graphs summarizing results from experiments demonstrating that hepatitis C virus replicons cultured separately (top) have similar sensitivity to  $\alpha$ -interferon as hepatitis C virus replicons cultured with Sindbis virus replicons and yellow fever virus replicons (bottom).

#### Detailed Description of the Invention

**[ 0011]** The present invention provides novel methods and compositions useful for evaluating candidate antiviral agents for antiviral activity. The methods comprise simultaneously evaluating the antiviral activity of the agent on more than one subgenomic viral replication system. In these methods, each subgenomic viral replication system is able to replicate in the cells of a cell culture so that the antiviral activity of the candidate antiviral agent is evaluated by applying the agent to the cell culture and determining the effect of the agent on replication of the subgenomic viral replication system in the cell culture.

**[ 0012]** As used herein, a subgenomic viral replication system is an incomplete viral genome capable of replication, but lacking in one or more

genetic elements that are essential for producing infectious virus particles.

**[ 0013]** There are two types of subgenomic viral replication systems: replicons and defective genomes (minigenomes).

**[ 0014]** A viral replicon is a subgenomic viral replication system, derived from a viral genome, that is capable of replicating within cells cultured *in vitro* (Agapov et al., 1998). They typically encode all of the *cis*- and *trans*-acting viral components required for replication and transcription of the viral genome within a cell, but lack one or more functional element required for full virus replication. The element could be lacking due to a deletion of all or part of the sequence encoding that function, or the element could be lacking due to a mutation, such as a point mutation, rendering the element nonfunctional.

**[ 0015]** Recently several reports have described the selection of replicons capable of persistent replication in cells (Frolov et al., 1999). Thus, cell lines can be created that contain persistently replicating viral replicons for a number of viruses. Table 1 provides a partial list of viruses where replicons have been made or could be made without undue experimentation.

Table 1. Viral replicons for antiviral screening

| Family                  | Virus (common names)               | Infectious clone | Noncytopathic replicon |
|-------------------------|------------------------------------|------------------|------------------------|
| <u>Togaviridae</u>      | Sindbis                            | yes              | yes                    |
|                         | Venezuela encephalitis virus       | yes              | possible               |
|                         | Rubella                            | yes              | possible               |
| <u>Picornaviridae</u>   | Poliovirus                         | yes              | yes                    |
|                         | Coxsackirus                        | yes              | possible               |
|                         | Enterovirus                        | yes              | possible               |
|                         | Hepatitis A                        | yes              | possible               |
| <u>Flaviviridae</u>     | Yellow fever                       | yes              | yes                    |
|                         | Dengue fever                       | yes              | possible               |
|                         | West Nile virus                    |                  | possible               |
|                         | Japanese Encephalitis virus        | yes              | yes                    |
|                         | Hepatitis C virus                  | yes              | yes                    |
|                         | Tick-born encephalitis virus (TBE) |                  | possible               |
| <u>Astroviridae</u>     | Astrovirus                         | yes              | possible               |
| <u>Rhabdoviridae</u>    | Rabies virus                       | yes              | possible               |
| <u>Orthomyxoviridae</u> | Influenza virus A                  | yes              | possible               |
|                         | Influenza virus B                  |                  | possible               |
| <u>Paramyxoviridae</u>  | Respiratory syncytial virus (RSV)  | yes              | possible               |
|                         | Measles                            | yes              | possible               |
|                         | Mumps                              | yes              | possible               |
| <u>Filoviridae</u>      | Ebola                              | yes              | possible               |
|                         | Marburg                            |                  | possible               |
| <u>Bunyaviridae</u>     | La Crosse virus                    |                  | possible               |
|                         | California encephalitis virus      | yes              | possible               |
|                         | Hantaan virus                      |                  | possible               |
|                         | Crimean-Congo                      |                  | possible               |
|                         | Rift Valley fever                  |                  | possible               |

| Family                  | Virus (common names)   | Infectious clone  | Noncytopathic replicon   |
|-------------------------|--|---|--|
| <u>Arenaviridae</u>     | Lassa fever<br>Argentine Hemorrhagic fever<br>Bolivian Hemorrhagic fever   |   | possible<br>possible<br>possible   |
| <u>Reoviridae</u>       | Colorado tick fever  |   |  |
| <u>Hepadnaviridae</u>   | Hepatitis B virus  | yes   | yes  |
| <u>Papillomaviridae</u> | Human papilloma virus  | yes   | yes  |
| <u>Polyomaviridae</u>   | JC virus<br>BK virus   | yes<br>yes  | possible<br>possible   |
| <u>Herpeviruses</u>     | Herpes simplex virus type one (HSV-1)<br>Herpes simplex virus type two (HSV-2)<br>Epstein-Barr virus (EBV)<br>Human cytomegalovirus (HCMV)<br>Varicella-zoster virus (VZV)<br>Human herpes virus type six (HHV6)<br>Human herpes virus type seven (HHV7)<br>Human herpes virus type eight (HHV8) | yes<br>yes<br>yes<br>yes<br>yes<br>possible<br>possible<br>possible | yes<br>possible<br>yes<br>possible<br>possible<br>possible<br>possible<br>possible |
| <u>Adenoviridae</u>     | Human adenovirus   | yes   | possible   |
| <u>Retrovirus</u>       | Human immunodeficiency virus type one (HIV-1)<br>Human immunodeficiency virus type two (HIV-2)<br>Human t-cell leukemia virus type one (HTLV-1)<br>Human t-cell leukemia virus type two (HTLV-2)   | yes<br>yes<br>yes<br>yes  | possible<br>possible<br>possible<br>possible                                       |
| <u>Parvoviridae</u>     | Human parvovirus<br>Adeno-associated virus   | yes<br>yes  | possible<br>yes  |



**[ 0016]** Cell cultures comprising replicons offer a number of benefits in discovery and analysis of antiviral agents. They permit the effect of an antiviral agent to be observed in the context of living cells, so that any agents that show antiviral activity necessarily enter and act within living cells. Replicon-containing cell cultures also allow the immediate identification of antiviral agents with obvious undesirable cytotoxicity using well established cytotoxicity assays. These cell cultures also permit cell-based drug discovery screens and other studies to be performed against viruses such as hepatitis C virus (HCV) and human papillomavirus (HPV) that are unable to be conventionally cultured *in vitro*. Since viral functions related to infectivity are typically not required for viral genome replication, viral replicons lacking at least one infectivity-related sequence are much safer and thus easier to work with than infectious virus. As used herein, an infectivity-related sequence is a sequence required for the virus to infect a cell.

**[ 0017]** Another advantage of the replicon-containing cell cultures is that the replicons can be genetically manipulated to comprise heterologous sequences such as those encoding reporter genes such as luciferase, beta-galactosidase, secreted alkaline phosphatase or green fluorescent protein (MacDonald and Johnson, 2000) that facilitate high throughput automated analysis of viral genome copy number (Frolov et al., 1996).

**[ 0018]** An alternative approach to developing non-infectious viral replication systems is to use defective genomes. Defective viral genomes contain all the *cis*-acting elements required for viral genomic replication and transcription, but lack one or more of the genetic elements that encode the *trans*-acting factors required for replication. Such defective genomes, therefore cannot replicate by themselves (i.e. they are not replicons), but they can be replicated if the missing factor or factors are supplied *in trans*. A cell that contains the defective genome plus the necessary *trans*-acting factors exhibits a functional similarity to a replicon in that partial viral replication occurs within the cell and no infectious virus is produced. As with cell cultures containing

replicating replicons, cell cultures containing replicating defective viral genomes represent a useful tool for antiviral drug discovery. Examples of defective genomes include the genomes contained within defective interfering virus particles that have been observed for many RNA and DNA viruses such as Alphaviruses (e.g. Sindbis virus) and herpesviruses (e.g. herpes simplex virus type one), respectively.

**[ 0019]** Another example of a defective genome is a minigenome. This is a type of artificial genome that has been constructed for a number of negative-strand RNA viruses such as respiratory syncytial virus (RSV), rabies virus, measles virus, etc. A minigenome of these viruses is an incomplete genome that contains all the *cis*-acting sequence elements that are required for replication of the viral RNA genome, but lack one or all of the coding regions of the complete viral genome.

**[ 0020]** Another example of defective viral genomes are so-called amplicons of DNA viruses such as herpesviruses (e.g. HSV-1), and other DNA viruses such as Papovaviruses (e.g. simian virus 40 (SV40)). Such amplicons are circular DNA molecules that contain the viral origin(s) of replication and are replicated within cells by the *trans*-acting replication factors (origin-binding proteins, replication enzymes, etc.) required by the virus.

**[ 0021]** A screening process that utilizes these subgenomic viral replication systems can identify inhibitors of any biochemical pathway involved in viral genome replication and transcription of viral genes. One need only select a screening procedure that evaluates the effects on the pathway of interest to be able to measure the effects of a candidate antiviral agent on that pathway. For example, to identify an agent that targets any pathway involved in replication, an end product of replication (for example the replicated genome) is measured after treatment with the agent by, e.g., performing quantitative PCR to quantify the amount of a representative portion of the genome that is present. Alternatively, to identify an agent that targets a specific pathway involved in

replication, e.g., translation of RNA polymerase, one could measure that particular component, e.g. quantifying RNA polymerase by an antibody assay.

**[ 0022]** The present invention provides methods and compositions for utilizing the advantages of subgenomic viral replication systems to screen candidate antiviral agents against multiple viruses simultaneously. By treating more than one subgenomic viral replication system with a candidate antiviral agent at the same time, candidate agents can be screened more rapidly. This multiple treatment approach also provides the ability to compare the effects of a candidate antiviral agent on each tested virus simultaneously. Thus, this system provides information on the specificity of the antiviral effect. This information is helpful, for example, in assessing whether the effect is acting on a specific viral target or on a cellular target and thus exerting its effect on the virus(es) indirectly. In addition this approach allows for the identification of compounds which exhibit broad antiviral activity and thus could be effective against many viruses.

**[ 0023]** In preferred embodiments of the invention, the cell cultures harboring subgenomic viral replication systems are combined into a multiple subgenomic replication culture (MSRC) also referred to as a mixed viral replicon culture (MVRC). This is possible because the replicons and minigenomes useful for the invention do not spread from cell to cell. Thus, each cell can be considered to be an autonomous unit, and determination of the effect of a candidate antiviral agent on each virus only requires that the various subgenomic viral replicon systems be distinguishable, e.g., by PCR methods or any other method that is capable of only amplifying a fragment of one of the subgenomic replication systems.

**[ 0024]** An MSRC offers a screening system in antiviral drug discovery that provides additional and distinct advantages over the use of cultures that only contain one subgenomic replication system. One advantage of using an MSRC for screening candidate antiviral agents is that it reduces the cost and labor required to analyze the effects of a large panel of candidate agents on sets of

distinct viruses. Another advantage of using an MSRC is that it simplifies the comparison of drug effects on distinct viral replicons as it insures that all replicons were exposed to the same quantity of compound under the same culture conditions.

**[ 0025]** Another advantage of using an MSRC for antiviral screening is that an MSRC system permits detection of antivirals that would be overlooked in a single virus screening system. In standard single-virus screening systems where antiviral agents affecting a single virus are generally identified by an antiviral effect above a threshold inhibition level. In those procedures, antiviral agents with sub-threshold levels of activity against the screened viral target are discarded. By screening a MSRC system, both specific inhibitors that target any one of the multiple targets and broad-spectrum agents that effect multiple viruses can be identified. Simultaneous recovery of inhibition data for a variety of distinct viral replicons thus permits identification of antiviral agents that might otherwise be overlooked.

**[ 0026]** Although the creation, analysis, and advantages of using MSRCs in antiviral drug discovery have not been described, multiplexed viral detection assays that permit quantification of multiple viruses in a single sample are known and used primarily for diagnosis of viral infections. For example, quantitative reverse transcription-polymerase chain reaction (qRT-PCR) mediated detection assays for multiple viruses in a single sample have been described (Elnifro et al., 2000; Grondahl et al., 1999; Fan et al., 1998; Jungkind et al., 1996; Burgart et al., 1992). Molecular beacon-based hybridization probes have also been employed to detect multiple viruses in a single sample (Vet et al., 1999). Multiplex assays for distinct gene reporters that can be independently assayed from the contents of a single culture well have also been described (Parsons et al, 2000; Grentzmann et al, 1998). While multiplexed viral detection assays may facilitate analysis of the effect of a candidate antiviral agent on an MSRC, they do not anticipate MSRCs or make them obvious, or teach how to develop antiviral screening methods using MSRCs, because they

do not utilize the advantages afforded with subgenomic viral replication systems. Additionally, the multiplex assays previously described are also not required to practice the antiviral screening assays of the present invention which utilize MSRCs, since schemes for subdividing the contents of an MSRC into multiple single well assays can be easily devised.

**[ 0027]** Thus, in some embodiments, the present invention is directed to methods of screening a candidate antiviral agent for antiviral activity. The methods comprise the following steps:

- (a) preparing a first cell culture comprising cells containing a first subgenomic viral replication system, and a second cell culture comprising cells containing a second subgenomic viral replication system;
- (b) adding the candidate antiviral agent to each cell culture;
- (c) incubating the cell cultures under conditions and for a time sufficient to detect an antiviral effect by the candidate antiviral agent on the subgenomic viral replication systems; and
- (d) determining the effect of the candidate antiviral agent on each viral replication system.

**[ 0028]** Although some advantages are achieved from this invention even if the cells containing the subgenomic viral replication systems are separate while performing the method, it is preferred that the cultures are mixed together into an MSRC prior to step (b). In those embodiments, the MSRC can contain from two to ten or more subgenomic viral replication systems, provided the effect of the candidate antiviral agent on each individual subgenomic viral replication system can be discerned. It is also possible to have more than one subgenomic viral replication system in a cell (see Example 2).

**[ 0029]** Thus, since each cell is an independent unit harboring a subgenomic viral replication system, each cell can be considered to potentially provide independent information relating to the candidate antiviral agent. Therefore, the number of different subgenomic viral replication systems that can

be combined in an MSRC can be as high as the number of cells in the MSRC, provided methods to evaluate viral replication in each cell are available. An example of such a method is the provision of different reporter genes with each subgenomic viral replication system where each reporter gene, for example, provides a different fluorescent end product (using e.g., molecular beacons specific for each viral RNA, or different fluorescent proteins and/or fluorescent products of an enzyme encoded by the subgenomic viral replication system). Each cell can then be analyzed by quantifying the intensity of the fluorescence of the fluorescent moiety associated with the subgenomic viral replication system in that cell. Optionally, such data can be obtained from each cell, e.g. using a fluorescence activated cell sorter.

**[ 0030]** The MSRC can also comprise a control cell culture which does not contain a subgenomic viral replication system in order to directly measure the effect of the candidate antiviral agent on the cells themselves.

**[ 0031]** The cells useful for this invention are any cells that can be grown in culture and that are capable of harboring a subgenomic viral replication system. This would include cells from any eukaryotic family, including plants, fungi, insects, and protists. In most embodiments, the cells will be from the same organism that is capable of harboring the virus of interest. In preferred embodiments, the cells are animal cells, preferably mammalian cells; in most preferred embodiments, the cells are human cells.

**[ 0032]** The cells useful for these methods can be either primary cells or from an established cell line. When grown together in a MSRC, the cells must have similar growth conditions such that each cell will support replication of the subgenomic viral replication system that it contains.

**[ 0033]** The subgenomic viral replication system in the cells can be transient or stably maintained in the cells. It is only required that the subgenomic viral replication system be able to replicate for sufficient time to be able to evaluate whether replication is inhibited by the candidate antiviral agent.

**[ 0034]** Each subgenomic viral replication system used in the methods and compositions of the present invention must be genetically distinct from the other subgenomic viral replication system(s) evaluated. The subgenomic viral replication systems can be in different virus families or same family, they can be the same virus but different genotypes, or even different mutants of same virus. The only requirement is that they must be distinguishable from each other in the embodiments where the two cell cultures are mixed together. In some embodiments, the subgenomic viral replication systems can differ by as little as one basepair. Such a method could be useful, for example, in determining whether a particular protein that is mutated in one subgenomic viral replication system, but not the other system, is the target of the candidate antiviral agent.

**[ 0035]** The methods of the present invention are useful for any virus that can be made into a subgenomic viral replication system, including DNA viruses, RNA viruses, retroviruses and viroids. It is also envisioned that the invention methods would be adaptable for any virus that cannot now be made into a subgenomic viral replication system but could be made into such a system in the future.

**[ 0036]** The viruses useful for this invention includes any virus from which a subgenomic viral replication system can be derived. This would include viruses that infect the cells of organism from any eukaryotic kingdom, phyla, or family, including plants, fungi, insects, and protists. In preferred embodiments, the virus infects animal cells, preferably mammalian cells; in most preferred embodiments, the virus infects human cells. Examples of preferred viruses are included in Table 1, and include hepatitis C virus, yellow fever virus, respiratory syncytia virus and Sindbis virus, as utilized in the Examples.

**[ 0037]** Any candidate antiviral agent can be evaluated using the methods of the present invention, including any small to large organic or inorganic molecule, provided the agent can be taken up by the cells when added to the culture. To assist in this uptake, the candidate antiviral agent can be formulated into compositions comprising excipients such as liposomes,

amphipathic compounds etc., as is well known in the art. The cells harboring the subgenomic viral replication system can also be treated, e.g., with polyethylene glycol or with an electroporation device, to assist in the uptake of the candidate antiviral agent.

**[ 0038]** Nonlimiting examples of candidate antiviral agents include nucleotides or nucleosides (e.g., AZT); oligonucleotides or polynucleotides such as antisense compounds, vectors comprising antisense or genes encoding antiviral proteins, including plasmids, viral vectors, etc. Nucleic acid mimics such as peptide nucleic acids (see, e.g., Corey, 1997), and other nucleic acid binding compounds, (e.g., those described in Geierstanger et al., 1996) can also be utilized as candidate antiviral agents. Additionally, secondary metabolites and other small chemicals, bioactive amino acids, oligopeptides or polypeptides can be tested for antiviral activity, including antibodies and compounds comprising antibody binding sites, enzymes, structural proteins, growth factors, transcription factors, etc.

**[ 0039]** After adding the candidate antiviral agent to the cell culture, the cells are incubated under conditions and for a time sufficient to detect an antiviral effect by the agent. Depending on the virus, what is being measured to evaluate antiviral activity, and the nature of the cells harboring the subgenomic viral replication system, the optimum time of incubation could be anywhere from 1 minute to 48 hours, or longer. The shorter time periods are envisioned, for example, where the antiviral activity of the candidate antiviral agent is measured by evaluating whether the agent binds to, e.g., a particular protein, such as an enzyme, where inhibition of enzyme activity, or binding of the candidate agent to the enzyme, is measured.

**[ 0040]** When the antiviral activity of the candidate antiviral agent is measured by determining whether replication has been inhibited by the agent, the cell culture must be incubated long enough to allow a measurable amount of replication to occur, but not so long that the cells overgrow the culture dish. With virus/cell combinations such as those used in the Examples below



(hepatitis C virus replicon in Huh7 hepatocytes; Sindbis virus replicon in BHK kidney fibroblasts; yellow fever virus replicon in Huh7 cells; respiratory syncytia virus minigenome transiently expressed in the above-described BHK cells harboring the Sindbis replicon), 20-24 hours incubation was determined to be optimum.

**[ 0041]** In some aspects of the invention, the antiviral effect of the candidate antiviral agent is determined by an assessment of the amount of replication that has occurred. Nonlimiting examples of methods for making these determinations include any method of RNA or DNA quantitation that involves target amplification, such as quantitative RT-PCR or PCR or transcription mediated amplification; or non-amplification DNA or RNA quantitation methods, particularly those that involve signal amplification such as branched chain DNA, but also northern or Southern hybridization, in-situ hybridization, molecular beacons, etc. To ascribe copy numbers of replicon, comparison with RNA standards can be made.

**[ 0042]** For any particular subgenomic viral replication system, the appropriate assay to measure viral replication would be within the ability of a skilled artisan to determine without undue experimentation.

**[ 0043]** The activity of a candidate antiviral agent can also be determined by methods that target a particular component of the virus required for replication. Such assays might be desirable, for example, if the candidate antiviral agent was an antisense oligonucleotide that binds to a gene encoding a protein such as an enzyme essential for viral replication. For these targeted assays, any method for quantitation of the specific protein could be useful, for example immunoassays such as EIA, ELISA, immunoblotting, immunofluorescence or immunoprecipitation; assays for enzymatic activity of a particular viral enzyme, for example RNA or DNA polymerase, protease, helicase, thymidine kinase, ribonucleotide reductase, etc.; or assays of products of reporter genes that have been fused to a particular viral protein or otherwise inserted into the genome. Nonlimiting examples of these reporter proteins

include luciferase, green fluorescent protein, and  $\beta$ -galactosidase.

**[ 0044]** In other embodiments, the present invention is directed to mixed cell cultures comprising a first cell culture comprising cells containing a first subgenomic viral replication system and a second cell culture comprising cells containing a second subgenomic viral replication system. These were previously named multiple subgenomic replication cultures or MSRCs. Any of the mixed cell cultures that are useful for the methods of screening candidate antiviral agents as described above are envisioned to be within the scope of these embodiments. Included are mixed cell cultures harboring any number of subgenomic viral replication systems up to the number of cells in the culture, for example three, four, five, ten, twenty or more independent subgenomic viral replication systems.

**[ 0045]** Preferred embodiments of the invention are described in the following Examples. Other embodiments within the scope of the claims herein will be apparent to one skilled in the art from consideration of the specification or practice of the invention as disclosed herein. It is intended that the specification, together with the examples, be considered exemplary only, with the scope and spirit of the invention being indicated by the claims which follow the examples.

#### Example 1. Multiplex Screening of HCV/YFR/SINDBIS in Mixed Viral Replicon Culture

**[ 0046]** To determine if the effects of well characterized antiviral agents could be observed in an MVRC, cell lines containing HCV, yellow fever, and Sindbis viral replicons were cultured both independently and as mixed cultures in the presence and absence of fixed amounts of  $\alpha$ -interferon ( $\text{IFN}\alpha$ ), a well characterized antiviral agent (Constantinescu, 1991). Interferon has previously been shown to inhibit replication of HCV both in vivo and in vitro (Podevin et al, 2001; Stewart and Sheaff, 1969; Ryman et al., 2000; Saito, 1990; Arroyo et al., 1988). Following exposure to the antiviral agent, RNA was extracted from

the cells and the levels of viral replicon determined via a qRT-PCR assay.

## Materials and Methods

**[ 0047]     Viral Replicon Cultures.** The Sindbis viral replicon system referred to as BHK/SR19/pac and the HCV replicon Ava.5 cell lines were generated as described previously (Frolov et al., 1999; Blight et al., 2000; Bredenbeek et al., 1993; Rice et al, 1987). The yellow fever virus (YFR) replicon-containing Huh7 cells were made using a similar strategy (Rice et al., 1989).

**[ 0048]     Cells** were seeded in standard 12-well microtiter dishes (appx. 5 cm<sup>2</sup>) with the indicated levels of the antiviral agents  $\alpha$ -interferon (Sigma, St. Louis, MO) in minimal essential media (MEM, Life Technologies, Gaithersburg, MD) plus 10% fetal bovine serum and incubated at 37°C under standard conditions. The total number of cells seeded to each well was 200,000. When the three cultures were mixed, the relative proportions of each were 30% HCV/40% Sindbis/30% YFR replicon containing cells.

**[ 0049]     qRT-PCR Analysis.** RNA from the cell cultures was prepared via a commercially available kit (RNAeasyJ, Qiagen, Valencia, CA). RNA was subsequently added to a qRT-PCR aqueous cocktail (50  $\mu$ L) consisting of 10  $\mu$ L 5X EZ Buffer, 25  $\mu$ L 10X manganese acetate, 3  $\mu$ L of each primer at 5  $\mu$ M, 1.5  $\mu$ L of each dNTP at 10 mM; 0.5  $\mu$ L of uracil-N-glycosylase (as supplied); 2.0  $\mu$ L of rTth Polymerase (2.5 u/1  $\mu$ L) (EZ rTth RNA PCR Kit, Perkin Elmer, Boston, MA) and 5 uL of a 1:10,000 dilution of SYBR GreenJ Fluorescent Dye as supplied (SYBR GreenJ, Molecular Probes, Eugene, OR).

**[ 0050]     Primers** for amplification of the HCV 3' NTR sequence are: 5' - ggctccatcttagccctagtc (SEQ ID NO:1) and 3' - agtatcggcactctctgcagt (SEQ ID NO:2). The YFR replicon was identified by the primers: 5' - ggatgcaggtaccactagaa (SEQ ID NO:3) and 3' - cgtggtgatctggttgatt (SEQ ID NO:4). The Sindbis replicon was identified by the primers: 5' - gagagcgccacgtagtgta (SEQ ID NO:5) and 3' - accttgactgctcctcttc (SEQ ID NO:6).

**[ 0051]** Real time fluorescence monitoring of the qRT-PCR reaction was accomplished and assignment of the threshold PCR amplification cycle (i.e. the thermal reaction cycle at which detectable exponential amplification of the reaction product is observed) via use of a BioRad I-cycler instrument. Experiments with dilutions of RNA target standards at known concentrations confirm that the threshold cycle is an accurate measure of viral replicon levels. The threshold cycle value for the no interferon control was arbitrarily set to 100% (i.e. 100% of control) with values in the presence of interferon expressed as a percentage of the negative control.

#### Results

**[ 0052]** Table 2 shows the results of an experiment using real time quantitative polymerase chain reaction (qRT-PCR) to measure the effect of IFN $\alpha$  on three viral replicons assayed in a mixed viral replicon culture. Since each replicon-containing cells were all exposed to IFN $\alpha$  under exactly the same conditions, the relative effect of IFN $\alpha$  on distinct replicons is easily determined. In this example it is readily apparent that the inhibitory effects of IFN $\alpha$  are most pronounced in Sindbis (>98% inhibition at 100u/ml IFN $\alpha$ ), followed by HCV (>91% at 100u/ml) and YFR (>81% at 100u/ml). These results are very similar to the effect of IFN $\alpha$  on each viral replicon when cultured separately (Figures 1, 2 and 3).

**[ 0053]** When ribavirin was tested in the mixed cultures, no effect was observed on HCV replication, even at the highest concentration tested (100  $\mu$ g/ml) (Table 3). However, ribavirin did inhibit Sindbis replicon replication in the mixed cultures. This further shows that antiviral agents act independently on the various subgenomic viral replication systems in multiple subgenomic replication cultures.

|                            | HCV<br>Mixed Viral<br>Replicon<br>Culture |       | Sindbis<br>Mixed Viral<br>Replicon<br>Culture |       | YFR<br>Mixed Viral<br>Replicon<br>Culture |       |
|----------------------------|---|-------|---|-------|---|-------|
| IFN $\alpha$<br>(Units/ml) | % of Control<br>(ave)                     | %CV   | % of Control<br>(ave)                         | %CV   | % of Control<br>(ave)                     | %CV   |
| 0                          | 100.00                                    | 31.06 | 100.00  | 38.47 | 100.00                                    | 43.67 |
| 1                          | 28.10                                     | 10.50 | 14.88   | 5.39  | 41.47                                     | 24.17 |
| 10                         | 14.94                                     | 5.92  | 8.53  | 4.41  | 26.09                                     | 12.95 |
| 100                        | 8.43                                      | 1.95  | 1.49  | 0.22  | 18.64                                     | 13.19 |

Table 2. Levels of replicon RNA of co-cultured hepatitis C virus (HCV), Sindbis virus, and yellow fever virus replicons following  $\alpha$ -interferon (IFN $\alpha$ ) treatment.

|                             | HCV<br>Mixed<br>Viral<br>Replicon<br>Culture |       | Sindbis<br>Mixed Viral<br>Replicon<br>Culture |       |
|-----------------------------|--|-------|---|-------|
| Ribavirin<br>( $\mu$ g/ml)  | % of<br>Control<br>(ave)                     | %CV   | % of Control<br>(ave)                         | %CV   |
| 0                           | 100.00                                       | 4.55  | 100.00  | 11.23 |
| 0.5                         | 100.37                                       | 6.50  | 40.54   | 8.20  |
| 1                           | 101.51                                       | 5.34  | 62.98   | 12.34 |
| 10                          | 99.75  | 5.56  | 35.04   | 8.04  |
| 100                         | 110.22                                       | 3.76  | 18.11   | 1.28  |
| IFN- $\alpha$<br>(Units/ml) |  |       |   |       |
| 0                           | 100.00                                       | 35.04 | 100.00  | 12.81 |
| 100                         | 4.59   | 1.89  | 47.58   | 14.34 |
| 1000                        | 1.74   | 0.10  | 16.22   | 3.80  |

Table 3. Comparison of ribivirin and interferon effects on HCV and Sindbis replicon replication in mixed viral replicon culture.

**Example 2. Multiplex Screening of Stable and Transient Viral Replicons:  
HCV/RSV/Sindbis in Mixed Culture**

**[ 0054]** In Example 1, the feasibility of assaying multiple viral replicons cultured in a single well was demonstrated. Each of the viral replicons analyzed in the Example 1 is capable of autonomous replication within a cell line where it is stably maintained. However, there are other subgenomic viral replication systems that are not stably maintained. These systems require the use of a transient cell culture where the viral genome is introduced into the cells via transfection or electroporation. Once introduced, these transient replicons or minigenomes will display viral protein-mediated sub-genomic replication for a limited time before cells lacking viral sequences proliferate and predominate within the culture. There is clear value in being able to analyze the effect of antiviral agents on transient replicon or minigenome systems since stable viral replicon or minigenome systems have yet to be established for certain viruses.

**[ 0055]** Respiratory syncytia virus (RSV) is one example of a virus for which only a transient minigenome system is currently available. To determine if a multiple subgenomic replication culture containing both stable and transient subgenomic viral replication systems could be used to assay the effects of antiviral agents, stable HCV replicon-containing cells were mixed with cells containing a stable Sindbis replicon that had been transfected with a transient RSV minigenome system. This multiple subgenomic replication culture was subjected to both ribavirin and  $\alpha$ -interferon treatment followed by qRT-PCR analysis of replicon/minigenome levels.

**Materials and Methods**

**Viral Cultures.**

**[ 0056]** The Sindbis viral replicon system referred to as BHK/SR19/T7/pac and the HCV replicon Ava.5 cell lines were generated as described previously. The RSV minigenome system consisted of the following five plasmids: C2-LUC RSV minigenome cDNA (Collins et al, 1996); pTM1-N, pTM1-P, pTM1-L

(Grosfeld et al, 1995); and pM2-1. The pM2-1 vector was constructed by amplifying RSV (strain 2) M2-1 cDNA by reverse transcription PCR (RT-PCR) with the primers ccaaggatattgtcagg (SEQ ID NO:7) and ggggcaaataatgtcacgaaggaatcc (SEQ ID NO:8). The PCR product was cloned into pCR-Blunt (Invitrogen), cut out with EcoRI and cloned into the EcoRI site of the T7 expression vector pMH4. The pMH4 expression vector was derived from pTM1 (Elroy-Stein et al., 1989; kindly provided by Bernhard Moss, National Institutes of Health) by removal of the NcoI site.

**[ 0057]** To generate the cell line BHK/SR19/T7/pac/C2-LUC that transiently expresses the RSV minigenome C2-LUC,  $3 \times 10^6$  BHK/SR19/T7/pac cells were seeded into 10 cm<sup>2</sup> dishes. After 16 h the cells were transfected with 6 µg of C2-LUC, 1.2 µg pTM1-N, 0.6 µg pTM1-P, 1.2 µg pTM1-L and 0.6 µg pMH4-M2-1 per 10 cm<sup>2</sup> dish using the transfection reagent lipofectamine (Gibco Invitrogen, Carlsbad, CA). After 5-6h the transfection media was replaced with new growth media. 16 h post transfection the cells were trypsinized, counted and seeded into a 96 well plate at  $8 \times 10^3$  cells /well for each of the two cell lines. BHK/SR19/T7/pac/C2-LUC, BHK/SR19/T7/pac and Ava.5 cells were seeded at  $8 \times 10^3$  cells /well and served as control groups. All cell groups were set up in the presence of various concentrations of ribavirin or  $\alpha$ -interferon. After 24 h the cells were harvested for RNA extraction. qRT-PCR analysis was as in Example 1.

## Results

**[ 0058]** As with the stable replicons (HCV, YFR and Sindbis), RSV transient minigenome cell cultures showed similar sensitivity to ribovirin and  $\alpha$ -interferon whether in a single viral culture or a mixed culture (Table 4).

|                                   | RSV<br>Mixed Viral<br>Culture |       | RSV<br>Single Viral<br>Culture |       |
|-----------------------------------|-------------------------------|-------|--------------------------------|-------|
| Ribavirin<br>( $\mu\text{g/ml}$ ) | % of Control<br>(ave)         | %CV   | % of Control<br>(ave)          | %CV   |
| 0                                 | 100.00                        | 43.21 | 100.00                         | 28.72 |
| 0.5                               | 96.16                         | 39.35 | 97.31                          | 8.30  |
| 1                                 | 44.14                         | 12.68 | 17.86                          | 3.93  |
| 10                                | 42.42                         | 14.55 | 3.74                           | 0.18  |
| 100                               | 13.90                         | 5.07  | 2.82                           | 0.53  |
| IFN- $\alpha$<br>(Units/ml)       |                               |       |                                |       |
| 0                                 | 100.00                        | 13.21 | 100.00                         | 13.21 |
| 100                               | 84.30                         | 33.61 | 84.30                          | 34.02 |
| 1000                              | 77.80                         | 56.54 | 47.29                          | 7.47  |

Table 4. Comparison of ribavirin and  $\alpha$ -interferon (IFN- $\alpha$ ) effects on RSV minigenome replication in mixed and single viral replicon culture.

[ 0059] In view of the above, it will be seen that the several advantages of the invention are achieved and other advantages attained.

[ 0060] As various changes could be made in the above methods and compositions without departing from the scope of the invention, it is intended that all matter contained in the above description and shown in the accompanying drawings shall be interpreted as illustrative and not in a limiting sense.

[ 0061] All references cited in this specification are hereby incorporated by reference. The discussion of the references herein is intended merely to summarize the assertions made by the authors and no admission is made that any reference constitutes prior art. Applicants reserve the right to challenge the accuracy and pertinence of the cited references.



SEQ ID Nos -

SEQ ID NO:1 - HCV 3' NTR 5' primer CB3N51

ggctccatcttagccctagtc

SEQ ID NO:2 - HCV 3' NTR 3' primer CM38B5

agtatcggcactctctgcagt

SEQ ID NO:3 - YFR 5' primer 2535

ggatgcaggtaccactagaa

SEQ ID NO:4 - YFR 3' primer 2638

cgtggtggatctggttgatt

SEQ ID NO:5 - Sindbis 5' primer SV1938

gagagcgccacgtagtgta

SEQ ID NO:6 - Sindbis 3' primer SV2044

accttgactgctctcttc

SEQ ID NO:7 - RSV M2-1 cloning primer 1

ccaaggatatttgcagg

SEQ ID NO:8 - RSV M2-1 cloning primer 2

ggggcaaatatgtcacgaaggaatcc

SEQ ID NO:9 - luciferase 5' primer

catcacgtacgcggaatact

SEQ ID NO:10 - luciferase 3' primer

cgcaactgcaactccgataa